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CTNNB1 Mutations and β-Catenin Protein Accumulation in Human Hepatocellular Carcinomas Associated With High Exposure to Aflatoxin B1

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β-Catenin plays a key role in the Wnt signaling pathway, and mutations of *CTNNB1,* the gene that encodes β-catenin, have been identified in about one-fourth of human hepatocellular carcinomas from regions of low aflatoxin B1 exposure. In this study 62 hepatocellular carcinomas (HCCs) from people highly exposed to aflatoxin B1 in Guangxi, People s Republic of China, were laser-capture microdissected and examined for *CTNNB1* mutations. In addition, 41 of the HCCs were evaluated for the presence of the β-catenin protein by immunohistochemical methods. Twenty of the HCCs showed positive results for β-catenin, with strong membrane staining, while adjacent non-neoplastic liver tissue lacked or showed only weak membrane staining. One HCC, in which a *CTNNB1* mutation was not detected, showed nuclear staining for the β-catenin protein. Mutations of *CTNNB1* were identified in five HCCs. These consisted of four point mutations in the glycogen serine kinase-3β phosphorylation region of codons 32 45 and one deletion of codons 32 38. These mutations were similar to those previously reported for human HCC, although at a lower frequency. A signature mutation profile associated with aflatoxin B1 exposure could not be identified. The immunohistochemical findings indicate a role for accumulation of β-catenin and possibly increased Wnt signaling in aflatoxin B1 associated HCC. The low frequency of *CTNNB1* mutations, however, suggests that mutation of another Wnt signaling component, such as the Wnt scaffolding protein axin or the adenomatous polyposis coli protein, both of which modulate β-catenin stability, also may be involved in aflatoxin-associated HCC. Published

Key words: hepatocellular carcinoma; a atoxin B1; *CTNNB1* mutations; β-catenin protein

INTRODUCTION

The high incidence rates of hepatocellular carcinoma (HCC) that occur in certain areas of Asia and Africa are associated with elevated levels of exposure to the aatoxin B1 mycotoxin and infection with hepatitis B virus [1]. Estimates of aatoxin B1 exposure levels in the Chinese provinces of Jiansu, Zhejiang, Fujian, Guangdong, and Guangxi are as high as 2027 ng/kg/day [2], compared with levels in the United States of <3 ng/kg/day [3]. Aatoxin-associated HCC is known to be correlated with a p53 ngerprint mutation, a G to T transversion at the third position of codon 249 that occurs in about 50% of tumors from patients exposed to high levels of aatoxin [4].

Upregulation of the Wnt signaling pathway by mutation of one of the critical members of the pathway appears to play an important role in the development of certain cancers, including colorectal and hepatocellular cancers and melanoma [5]. HCCs frequently have mutations in *CTNNB1,* the gene that encodes β-catenin. These mutations most often are localized to codons 32 45 of exon 3, a region that codes for glycogen serine kinase (GSK)-3β phosphorylation sites [6 9] and several ubiquitination sites critical for modulating β-catenin stability and Wnt signaling [10]. Mutations in *CTNNB1* were identied in eight of 31 (26%) [6], 14 of 75 (19%) [7], 21 of 119 (18%) [8], and 14 of 73 (19%) [9] of the HCCs examined in previous studies. Of the HCCs examined for

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Abbreviations: HCC, hepatocellular carcinoma; *CTNNB1,* human gene encoding β-catenin; GSK, glycogen serine kinase; APC, adenomatous polyposis coli; PCR, polymerase chain reaction; *Catnb,* mouse gene encoding β-catenin.

CTNNB1 mutations in those studies, none have been reported to come from patients living in areas with high levels of exposure to aflatoxin. There has been no evidence of specific patterns of *CTNNB1* mutations detected in the HCCs in those studies.

In this study we examined 62 HCCs associated with high levels of exposure to aflatoxin B1 for *CTNNB1* mutations in the GSK-3β target region sites most commonly mutated in cancer. Based on material availability, we examined a subset of 41 of the HCCs for altered accumulation of β-catenin protein. The cases were collected in Guangxi, People's Republic of China, where extremely high levels of aflatoxin B1 are found in foodstuffs [1]. These samples have been examined previously for *p53* codon 249 mutations and p53 protein expression [4].

METHODS

Sample Procurement

Paraffin-embedded formalin-fixed blocks containing HCCs from patients in southern Guangxi, China, were obtained (by Z.-Q. Z.) at the Cancer Institute of Guangxi. All patients resided in this region.

Immunohistochemistry

Immunohistochemical staining of β-catenin protein was performed as described previously [11] with a polyclonal goat anti–β-catenin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 on serial 6-μm sections. In place of the primary antibody, normal goat serum was used as the negative control. Slides were reviewed by a pathologist (G.P.F.), and immunochemical staining of the membranous and nuclear areas was scored by the ''quick multiplicative scoring'' technique of Detre et al. [12]. Staining intensities of 0–3 were multiplied by the score of the proportion of cells that stained positive (1 = 0–4%, 2 = 5–19%, 3 = 20–39%, 4 = 40–59%, 5 = 60–79%, and 6 = 8–100%). Because of background cytoplasmic staining in non-neoplastic areas in several of the negative controls, cytoplasmic staining was not scored.

Sample Microdissection and DNA Isolation

A laser capture microdissection instrument (Arcturus Engineering, Inc., Mountain View, CA) was used to dissect tumor tissue away from non-neoplastic tissue in several consecutive 10- to 15-μm-thick sections. Duplicate samples were obtained for each tumor and extracted separately. To obtain DNA, microdissected samples were digested in 50–200 μL of buffer containing 10 mM Tris-HCl, 1 mM EDTA, 1% Tween-20, and 0.04% proteinase K (Sigma Chemical Co., St. Louis, MO) and incubated overnight at 55°C. The samples were boiled and centrifuged to remove tissue debris, and the supernatant was removed and stored at −20°C until use. Because of the age and long fixation time of the tumor blocks, high-quality DNA was not obtained from many samples, limiting polymerase chain reaction (PCR) amplification to small amplicons. Thus, we were able to focus only on the GSK-3β target region for mutations in exon 3 of *CTNNB1*.

Mutation Detection and Identification

The part of exon 3 of *CTNNB1* that included the GSK-3β binding region of codons 32–45 was amplified with PCR. Seminested amplification reactions were performed with primers 1F (5′-CTG ATT TGA TGG AGT TGG AC-3′)+3R (5′-CCT CTT CCT CAG GAT TGC CT-3′) (152-bp product) for the outer reactions and 4F (5′-AGC GGC TGT TAG TCA CTG GC-3′)+3R (109-bp product) for the inner reactions, with [^{33}P]-dATP incorporated into the reaction. The three-step amplification cycling conditions included denaturation at 94°C, annealing at 53°C, and extension at 72°C for 25 cycles for the outer reaction and 94°C, 55°C, and 72°C for 30 cycles for the inner reaction. Normal human DNA and no DNA controls were included along with the tumor samples.

Screening for mutations was performed by single-strand conformation polymorphism analysis under two gel conditions: 6% acrylamide gels with 10% glycerol electrophoresed at 40 W constant power at 4°C and 0.5× MDE (FMC Bioproducts, Rockland, ME) gels electrophoresed at 3 W at room temperature. Aberrantly migrating bands in these gels were extracted, reamplified, purified, and cycle-sequenced with a Thermo-sequenase kit (US Biochemical, Cleveland, OH). Primers 4F and 3R were used as sequencing primers. Only HCCs with mutations identified in DNA from both independently extracted duplicate laser-capture–microdissected samples were scored as positive.

RESULTS

Forty-one of the aflatoxin B1–associated HCC samples were examined by immunohistochemistry for expression of the β-catenin protein (Figure 1 and Table 1), and 21 showed strong membrane staining, as assessed by a quick multiplicative score of 10–18 [12]. For 22 specimens, both non-neoplastic and tumor tissues from the same section were available for microscopic examination, and 14 of those samples (67%) showed intense cell membrane staining in tumor cells. In those 14 samples there was little or no membrane staining in adjacent non-neoplastic liver. Of the other eight samples, four had membrane staining scores of 10–18 in both tumor and nontumor areas, and four had almost no staining (scores ≤ 6) in both tumor and non-neoplastic areas. There was no evidence of nuclear or membrane background staining in the negative control goat serum, although there was some cytoplasmic staining in non-neoplastic tissues

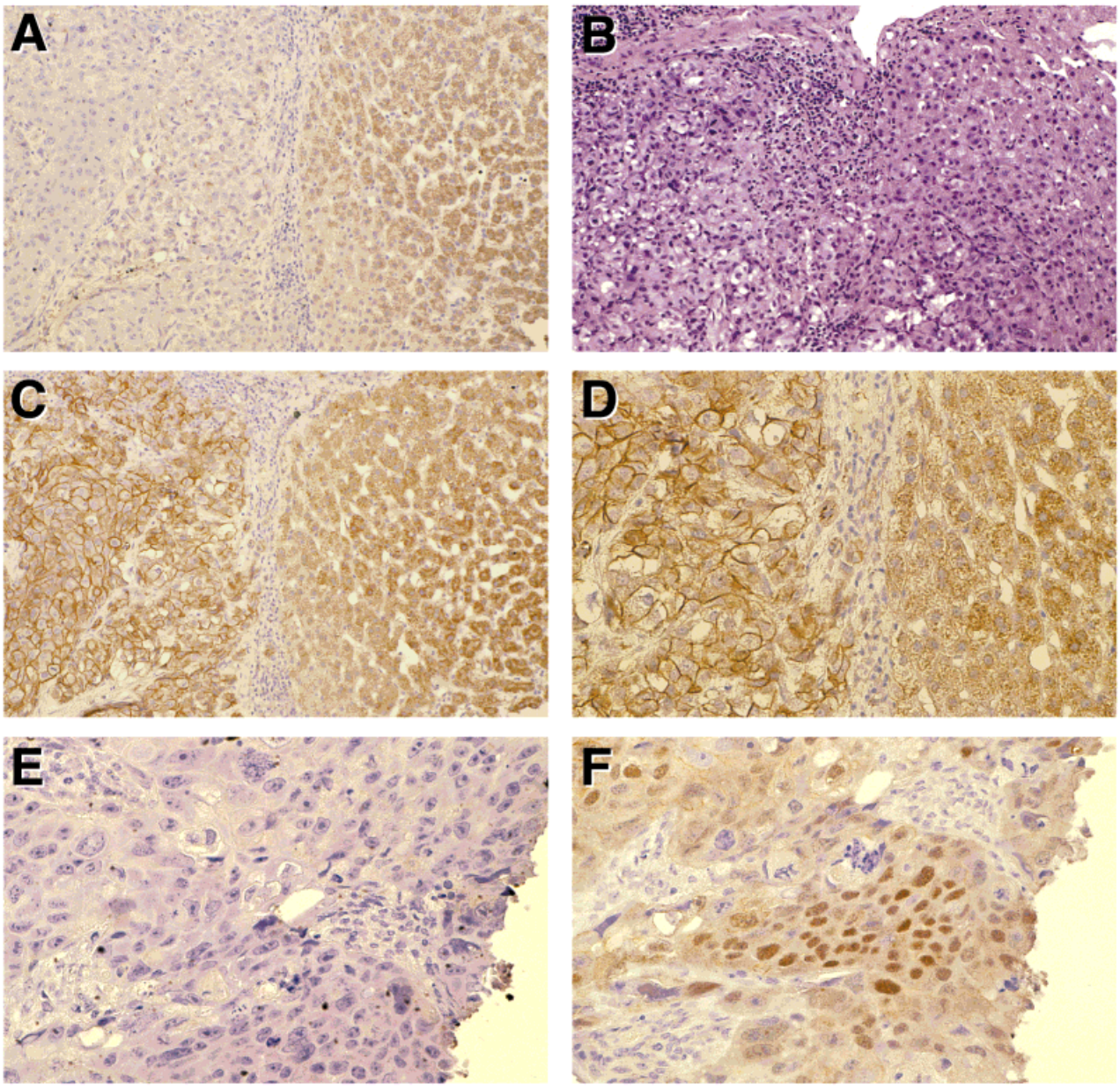

Figure 1. Immunohistochemical analysis of β-catenin expression in aflatoxin B1–associated human HCCs. (A) HCC on the left side of the panel and non-neoplastic tissue on the right (79 in Table 1) reacted with normal goat serum instead of primary β-catenin antibody (× 50). (B) The same sample as in A stained with hematoxylin and eosin (× 50). (C) The same sample as in A reacted with anti–β-catenin antibody (× 50). (D) Higher magnification (× 100) of the same sample, showing granular, nonspecific staining of cytoplasm in non-neoplastic liver (right) but strong membranous staining only in tumor cells (left). This was typical of other samples. (E) HCC (19 in Table 1) showing no nonspecific staining with normal goat serum–negative control (× 100). (F) The same sample as in E, showing areas of nuclear staining with antibody to β-catenin (× 100). This was the only HCC examined that showed nuclear staining.

(Figure 1A); for this reason, we did not score cytoplasmic staining. Only one HCC, which appeared to be invading the colon, showed positive nuclear staining (Figure 1E,F).

Sixty-two HCCs were analyzed for *CTNNB1* mutations in the GSK-3β phosphorylation region of exons 32–45. We identified five mutations: four point mutations and one in-frame deletion of codons 32–38 (Table 1 and Figures 2 and 3). The four point mutations were codon 32 **G**AC to **T**AC (Asp to Tyr), codon 33 T**C**T to T**G**T (Ser to Cys), codon 41 A**C**C to A**A**C (Thr to Asn), and codon 41 A**C**C to A**T**C (Thr to Ile). Each of these mutations has been reported for human HCCs in other studies [6–9]. Other PCR primer sets were designed to create larger products with which to detect deletion mutations, but they failed to provide reproducible PCR products.

Table 1. Summary of *CTNNB1* Mutations and β-Catenin Protein Detection by Immunohistochemistry in Aflatoxin-Associated Human Hepatocellular Carcinomas*

Sample	Age	Sex	β-catenin IHC	*CTNNB1* mutation	*p53* Codon 249 mutation
2	42	F	+	–	–
4	46	M	+	Codon 32 GAC to TAC	–
5	30	M	+	–	+
6	31	F	+	–	+
7	40	M	+	–	+
9	45	M	+	Deletion codons 32–38	–
10	04	M	+	–	–
11	36	M	+	–	–
13	23	M	+	–	–
14	50	F	+	–	–
16	57	F	+	Codon 41 ACC to ACC	–
19	58	M	+	–	ND
24	32	M	+	–	+
41	45	M	ND	Codon 41 ACC TO ATC	+
58	27	M	+	–	–
67	35	M	+	–	–
69	38	M	+	–	+
71	46	M	+	–	–
72	58	F	+	–	–
75	24	M	+	–	–
78	64	M	+	Codon 33 TCT to TGT	–
79	38	M	+	–	–

*IHC, immunohistochemistry; ND, not done or no results. Samples are the same as those used in Stern et al. [4]. Samples shown here stained positive by immunohistochemistry for β-catenin protein with a quick multiplicative score of 10–18 or had a mutation in *CTNNB1*. Any *p53* G to T mutation at codon 249 in these samples is also shown.

DISCUSSION

We detected *CTNNB1* mutations in about 8% of the aflatoxin-associated HCCs, whereas other reports have identified mutations in 20–25% of HCCs not associated with a particular exposure [6–9]. Among the mutations in the HCCs in these studies, large interstitial deletions involving exon 3 were detected in about 5% [6,7,9]. In our study we

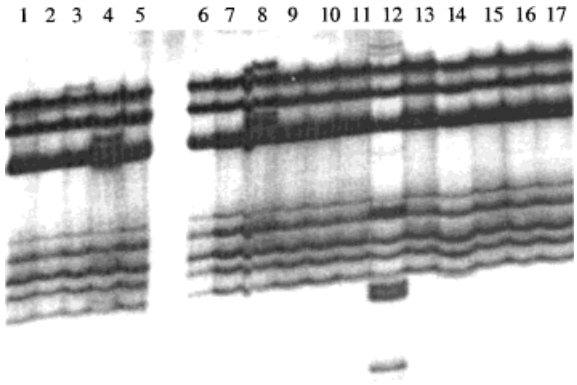

Figure 2. *CTNNB1* mutation detection by single-strand conformation polymorphism analysis in aflatoxin B1–associated HCCs. Lanes 1–5 and 7–17 contain HCCs and lane 6 normal human DNA. Mutations in the HCC samples in lanes 4, 8, and 12 were confirmed by sequencing.

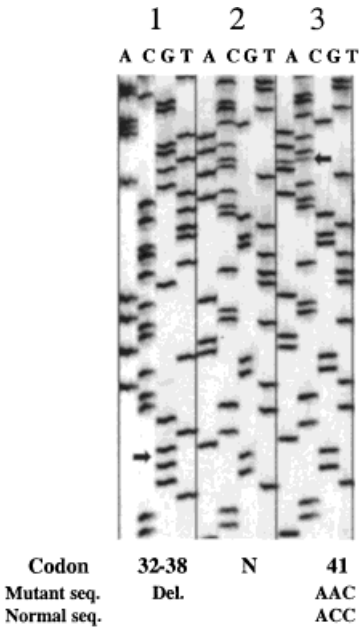

Figure 3. *CTNNB1* mutation identification by cycle sequencing of aflatoxin B1–associated HCCs. *CTNNB1* mutations were identified in sequence lanes 1 (deletion of codons 32–38) and 3 (codon 41, ACC to AAC). Arrows point to the beginning of the deletion at codon 32 in lane 1 and to a point mutation at the second base of codon 41 in lane 3. No detectable mutation in this region of exon 3 was found in lane 2.

assessed tumors for mutations only in the GSK-3β phosphorylation region. Had we been able to analyze larger portions of the gene, the frequency of mutations in the HCCs associated with aflatoxin exposure might have been higher. Testing for a correlation between the levels of aflatoxin B1 exposure and frequency of mutations was not possible, because it would take a much larger test population and individual measurements of exposure.

In the small number of *CTNNB1* mutations we did find in our study, there was no evidence of an aflatoxin B1–specific mutation pattern signature. Each of the mutations we identified, however, has been reported for other HCCs [6–9]. Each point mutation identified exhibited a different base change. This is unlike the ''fingerprint'' *p53* G to T mutation at codon 249 that appears to be associated with aflatoxin exposure [4,13]. We previously have reported a high frequency of mutations in the mouse *CTNNB1* homolog *Catnb,* the mouse gene encoding β-catenin, in certain chemically induced, but not spontaneous, hepatocellular neoplasms, although there was no evidence of chemical-specific mutation patterns [11]. Our hypothesis is that long-term exposure to non-genotoxic chemicals may cause oxidative stress and indirect DNA damage that leads to *Catnb* mutations in mouse livers. In humans, aflatoxin B1 is genotoxic in the liver and appears to target, in particular, the second G in codon 249 of p53. As in the mouse model, however, long-term exposure to aflatoxin B1 may cause indirect DNA damage to the liver that manifests in *CTNNB1* mutations that ultimately provide an alternative or additional selective growth advantage to the cell.

Even though we found a somewhat lower frequency of *CTNNB1* mutations in our HCCs, we noted increased β-catenin protein accumulation in about half of the samples analyzed, suggesting that upregulation of Wnt signaling is common in aflatoxin-associated HCC. The Wnt scaffolding protein axin and the adenomatous polyposis coli (APC) protein are two Wnt signaling members that modulate β-catenin stability, and these proteins are mutated in cancers in a tissue-dependent manner. Most colon cancers harbor a mutation in the *APC* gene, and a small number have mutations in *CTNNB1* [5]. Mutations in the axin1 gene have been identified in some HCCs that showed increased β-catenin protein accumulation without a *CTNNB1* mutation [14], suggesting that defects in other key regulatory elements can affect β-catenin stability and Wnt signaling. It is possible that high levels of aflatoxin exposure lead to a specific mutation in the APC, axin, or other Wnt signaling component in this pathway. As in our study, Terris et al. [9] identified tumor samples with β-catenin accumulation but no detectable *CTNNB1* mutation, providing additional evidence that other upstream members of the Wnt pathway, such as AXIN, may be mutated or dysregulated in HCC in ways that stabilize the β-catenin protein.

Only one aflatoxin B1–associated HCC showed positive nuclear staining (Figure 1E and F), and we did not detect a *CTNNB1* mutation in the GSK-3β binding region in that sample. However, we cannot exclude the possibility of a large deletion mutation outside the area amplified by our primers. Large deletion mutations have been identified previously in some colorectal carcinomas, hepatoblastomas, and chemically induced mouse liver tumors [6,15,16].

Unlike the immunohistochemistry data in our study, data reported by Terris et al. [9] showed nuclear accumulation of the β-catenin protein in 25% of HCC specimens. These researchers used a different antibody from the one we used, at a much higher titer. The staining pattern we saw is very similar to the pattern detected in chemically induced mouse hepatocellular neoplasms with strong membrane staining, but no nuclear staining [11]. Current hypotheses based on colon cancer models suggest that β-catenin protein accumulates and forms a complex with T-cell factor or lymphoid enhancer factor family members, which translocates to the nucleus. The β-catenin-bound complex activates the transcription of growth regulatory genes, which ultimately results in cell proliferation [17–19].

One possible explanation for not detecting nuclear staining of mutant β-catenin in most of the HCCs is that immunohistochemistry may not be the most sensitive measurement of Wnt signaling at the nuclear level. A small increase in nuclear β-catenin may effect a large increase in transcriptional activation. Another possibility is that the strong accumulation of β-catenin protein seen in the cell membranes of many HCCs affects processes such as cell adhesion in ways that may enhance liver carcinogenesis. One study on β-catenin protein expression in ovarian carcinomas reported that a subgroup of endometrioid carcinomas with exclusively membranous expression of β-catenin had a worse prognosis than other tumors that also showed nuclear staining [20]. We are now studying our mouse model *Catnb* mutation-positive HCCs for β-catenin binding partners and transactivation targets to understand the role of β-catenin accumulation in hepatocellular carcinogenesis. Despite the lack of nuclear staining of β-catenin in the mouse and human HCCs in our studies, the finding of increased β-catenin expression in the membranes of tumor cells, compared with normal liver, suggests the importance of this pathway in liver carcinogenesis.

These aflatoxin B1–associated HCCs also were examined for *p53* codon 249 G to T transversions, and this mutation was identified in 18 of 50 cases

[4]. Of the five tumors in which we detected *CTNNB1* mutations, one had both a mutation in *CTNNB1* and a *p53* codon 249 mutation. Moreover, six of the 21 cases in which we detected accumulation of β-catenin protein also had the *p53* mutation. This is not surprising, because, to our knowledge, β-catenin and p53 are not in the same pathway.

In this study we have provided evidence that increased Wnt signaling plays a role in aflatoxin B1–associated hepatocellular carcinogenesis. It seems probable that long-term exposure to aflatoxin B1 results not only in genotoxic damage to p53 in the liver but also in indirect DNA damage that leads to β-catenin accumulation, which enhances the carcinogenic process.

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